The ACIN1 Gene is Hypermethylated in Early Stage Lung Adenocarcinoma

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Introduction and Hypothesis: In recent years, many studies have performed genome-wide searching for differentially methylated genes in cancer. We hypothesized that characteristic aberrant hypermethylation of CpG islands of certain genes may exist in the early stages of lung adenocarcinoma and that such alterations may be useful in the detection and treatment of early lung adenocarcinoma.

Methods: A pair of immortalized cell lines originating from atypical adenomatous hyperplasia (PL16T) and from the resected end of the bronchus of the same patient (PL16B) was searched for aberrantly and differentially hypermethylated DNA fragments by a combination of the methylated CpG island amplification and suppression subtractive hybridization methods.

Results: From 229 clones, we selected 15 fragments that had a genomic region meeting the criteria for a CpG island. We identified a gene, apoptotic chromatin condensation inducer 1 (ACIN1), that was hypermethylated in PL16T. A higher frequency of hypermethylation at a locus at the 5′ end of the DNA fragment isolated from the ACIN1 gene was found in small-sized adenocarcinoma (2 cm or less) (30/37, 81%) compared with normal lung tissue (9/37, 24%, $p < 0.05$). Interestingly, hypermethylation of ACIN1 was detected relatively frequently in the normal counterpart of adenocarcinoma without bronchioloalveolar carcinoma (BAC) component (7/16, 44%), but was rare in the normal counterpart of adenocarcinoma with BAC component (2/21, 10%, $P < 0.05$).

Conclusions: We found hypermethylation of the ACIN1 gene in early stage lung adenocarcinoma. The role of methylation status in the development and malignant transformation of lung adenocarcinoma requires clarification.

Key Words: adenocarcinoma, methylation, ACIN1

(*J Thorac Oncol.* 2006;1: 160–167)

Lung cancer is one of the major causes of death in many countries. The total number of cancer deaths in Japan exceeds 300,000 per year, and mortality from lung cancer accounts for 20% of this total and is predicted to increase in the future. At the same time, 5-year survival rates of more than 80% are seen in patients with stage I lung cancer, and advances in early diagnosis and treatment are expected to improve these rates. Detection of cancer at earlier stages could potentially increase survival rates. Conventional methods of screening for lung cancer by chest X-ray and sputum cytology have proven ineffective in increasing survival rates,[1,2] so it is important to find more sensitive and specific diagnostic methods that can be used to detect the earliest stages of the disease.

One DNA-based alteration that commonly occurs in cancer is DNA methylation, an epigenetic modification of DNA. DNA methylation is a covalent chemical modification, resulting in a methyl (CH_3) group at the carbon-5 position of cytosine. CpG islands are CpG-rich areas of 0.5 to 5 kb in length that occur on average every 100 kb.[3,4] CpG islands are usually unmethylated. Approximately half of all human genes have CpG islands,[5] which are usually present in both housekeeping genes and tissue-specific genes.[6] Most studies performed in this area have examined individual aberrant methylated genes associated with cancer. Increased methylation in the promoter region of a gene leads to reduced expression, whereas methylation in the transcribed region has a variable effect on gene expression.[7,8,9] DNA methylation in lung cancer has been widely studied. To date, more than 30 genes have been found to have hypermethylation in their promoter region in lung cancer.[9,10] Hypermethylation of the APC, CDKN2A, CHD13, RARB, and RASSF1A genes is found in over 30% of patients with lung cancer, as established by at least two independent studies.[10] However, methylation does not guarantee gene silencing.[11] One reason for the great interest in DNA hypermethylation of CpG islands is its potential applicability as an epigenetic marker of cancer, and

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Submitted for publication

Accepted for publication

This work was supported in part by a Grant-in-Aid for Cancer Research (16-1) from the Ministry of Health, Labor and Welfare of Japan, and a Grant-in-Aid for Scientific Research (C) (17590292) from the Japan Society for the Promotion of Science.

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ISSN: 1556-0864/06/0102-0160

it has been suggested that the analysis of DNA methylation patterns could become a powerful tool for accurate diagnosis of early lung cancer.[10]

In recent years, many studies have involved genome-wide searching for differentially methylated genes in cancer. Toyota and colleagues applied methylated CpG island amplification (MCA) coupled with representational difference analysis (RDA) to identify novel CpG islands differentially methylated in tumors.[12–15] Ushijima and colleagues identified hypo- or hyper-methylated genomic fragments in tumors by a methylation-sensitive representational difference analysis (MS-RDA) method using the methylation-sensitive restriction enzyme *Hpa* II.[16–18]

We hypothesized that characteristic aberrant hypermethylation of CpG islands of genes may occur in the early stages of lung adenocarcinoma and that such alterations may be useful in the detection and treatment of early adenocarcinoma. The aim of this study was to isolate aberrantly methylated CpG islands in lung adenocarcinoma by genome-wide analysis and to identify the characteristics of lung cancer that are associated with methylation status. The initial search for aberrant methylation was performed with a pair of cell lines, PL16T, which was derived from atypical adenomatous hyperplasia (AAH), and PL16B, which was derived from the resected end of the normal bronchus in the same patient.[19]

MATERIALS AND METHODS

Tissue Samples, Cell Line And Histological Classification

A pair of cell lines, PL16T derived from AAH and PL16B derived from the resected end of the bronchus of the same patient who was 56-years old female and never smoker were used for the subtractive analysis for hypermethylated CpG islands.[19] Thirty-seven pairs of small-sized lung adenocarcinoma, less than 2 cm in diameter, and the adjacent noncancerous tissue were obtained from a bank of surgically resected material at the University Hospital of Tsukuba (Ibaraki, Japan) and Ibarakihigashi National Hospital (Ibaraki, Japan). All patients gave informed consent for specimen collection. The samples were from 22 male and 15 female patients (average age, 63.3 years; females, 62.6 years; males, 63.8 years). They underwent surgery during the period from April 1996 to October 2003. All tumor specimens were classified histologically according to the WHO histological classification and the histological criteria for small-sized lung adenocarcinoma proposed by Noguchi et al.[20] Noguchi et al. divided small adenocarcinomas into two groups. One is adenocarcinoma with replacement growth and the other is adenocarcinoma without replacement growth, which shows destructive growth at the margin of the tumor. As replacement growth indicates bronchiolo-alveolar spreading and/or lepidic growth, we divided the tumors examined in this study into adenocarcinoma with bronchioloalveolar carcinoma (BAC) component and adenocarcinoma without BAC component. All of the surgically resected specimens were fixed with formalin and embedded in paraffin. Additional methanol-fixed specimens and fresh frozen materials were prepared for the DNA and RNA analyses. Genomic DNA was extracted by standard phenol/chloroform procedures from the PL16T and PL16B cell lines and the tissue specimens. Total RNA was extracted from each frozen tissue or cell line with Trizol (Invitrogen Corp., Carlsbad, CA).

Methylated Cpg Island Amplification (MCA) Of The Pl16t And Pl16b Cell Lines

MCA was performed as described by Toyota et al.[13] Briefly, 5 μg of DNA extracted from the PL16T or PL16B cell lines was first digested with *Sma* I, which is a methylation-sensitive restriction enzyme, then digested again with the methylation-insensitive *Sma* I isoschizomer *Xma* I (New England Biolabs, Inc., Beverly, MA). The restriction fragments (0.5 μg) were ligated to RXMA12/24 (RXMA24: 5′-AGCACTCTCCAGCCTCTCACCGAC-3′; RXMA12: 5′-CCGGGTCGGTGA-3′) or RMCA 12/24 (RMCA24: 5′-CCACCGCCATCCGAGCCTTTCTGC-3′; RMCA12: 5′-CCGGGCAGAAAG-3′) adaptors with T4 ligase (New England Biolabs, Inc.) and amplified by PCR.

Modified Suppression Subtractive Hybridization (SSH) Between Pl16t And Pl16b Mca Amplicons

SSH was originally developed as a PCR-based method for cDNA subtraction analysis.[21,22] In this study, we compared two populations of DNA by this system (CLONTECH PCR-Select^{TM} cDNA Subtraction Kit; BD Bioscience Clontech, Palo Alto, CA, USA) with some modifications. In brief, MCA amplicons were digested with the restriction endonuclease *Sma* I to remove the RMCA 24mer adaptor or the RXMA 24mer adaptor and purified by Microcon YM-100 filtration (Millipore, Billerica, MA). Two-directional (forward and reverse) subtractive hybridization and unsubtractive hybridization were performed between PL16T and PL16B according to the manufacturer's instructions, and the subtractive hybridization products were amplified by PCR. First, diluted tester DNA (MCA amplicon of PL16T) was ligated to adaptor with T4 DNA ligase (both from the CLONTECH PCR-Select^{TM} cDNA Subtraction Kit). The subtraction consists of three steps. 1) First hybridization: an excess of driver DNA (MCA amplicon of PL16B) is hybridized with adaptor-ligated tester. 2) Second hybridization: an excess of fresh denatured driver DNA is mixed with adaptor-ligated tester DNA and hybridized. 3) PCR amplification: the first and second PCR amplifications are used to amplify exponentially molecules with different adaptor sequences, further reducing the background and enriching for differentially methylated sequences. Forward- and reverse-subtracted DNA and forward- and reverse-unsubtracted DNA were used as templates. The primer sequences used in the first PCR amplification were as follows: PCR primer 1, 5′-CTAATACGACTCACTATAGGG-3′. The primer 1 sequence was used as both forward and reverse primers. The first PCR products were diluted and subjected to a second PCR amplification. Primer sequences were as follows: nested PCR primer 1, 5′-TCGAGCGGCCGCCCGGGCAGGT-3′; nested PCR primer 2R, 5′-AGCGTGGTGCGGCCGAGGT-3′.

Cloning And Differential Screening For Ssh Products

The secondary PCR products (forward subtracted MCA amplicon of PL16T) were inserted into the pCR2.1 vector by use of a T/A cloning kit (Invitrogen Corp., Carlsbad, CA) and the plasmids were transformed into One Shot® DH5α-T1^R *E. coli* (Invitrogen Corp., Carlsbad, CA). The 2134 bacterial colonies were picked up and their insert DNA was amplified by PCR and blotted onto nylon membranes (Osmonics, Inc., Tokyo, Japan) and hybridized with forward- and reverse-subtracted and both unsubtracted probes. Then, a PCR-Select™ Differential Screening Kit (BD Bioscience Clontech) was used to screen clones with different DNA fragments inserted, according to the manufacturer's instructions.

Sequencing And Analysis

Two hundred and twenty nine clones were selected for sequence analysis. These insert fragments were sequenced with a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems Japan Ltd., Tokyo, Japan). Sequence homologies were identified with the BLAST program of the National Center for Biotechnology Information, available at http://www.ncbi.nlm.nih.gov/BLAST/.

Bisulfite Sequencing Of Acin1 Gene Clones

DNA was isolated by standard phenol-chloroform extraction and ethanol precipitation from surgically resected specimens of peripheral-type lung adenocarcinoma tissue and adjacent normal tissue from 37 patients. Genomic DNA was modified by treatment with sodium bisulfite.[23] We sequenced 3 to 5 clones from each of the specimens. The insert DNA fragments were amplified by PCR with Takara Ex Taq™ Hot Start polymerase (Takara Bio Inc., Tokyo, Japan). The primer sequence used in amplification of the ACIN1 gene was as follows: forward, 5′-ATTAAGATAGAGGAGTAGGGA-ATATT-3′; reverse, 5′-CCTACCTTTCTTCTCACTCT-TCTTT-3′. Then, the PCR products were ligated into the pCR2.1 vector (Invitrogen Corp., Carlsbad, CA) and the plasmid was transformed into One Shot® INVF'α *E. coli* (Invitrogen Corp.). Five clones for each sample were sequenced with a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems Japan, Ltd.).

Quantitative Real-time Rt-PCR Analysis

Total RNA was prepared with Trizol (Invitrogen Corp.) and 1μg samples were used for cDNA synthesis with random hexamers primer (TaqMan Reverse Transcription Reagents Kit; Applied Biosystems Japan, Ltd.). The primer sequences used were as follows: ACIN1-F, 5′-GGATCGGGACAAA-GTTCGAGA-3′; ACIN1-R, 5′-CAGTGGGAGCCAATA-GATGCAG-3′. Quantitative real-time RT-PCR was performed with SYBR Premix Ex Taq™ kit (Perfect Real Time; Takara Bio Inc.) and a Gene Amp 5700 sequence detection system (Applied Biosystems Japan, Ltd.). Numbers of target cDNA molecules were normalized to numbers of GAPDH cDNA molecules as a control.

Statistical Analysis

Associations of categoric variables were evaluated with Fisher's exact probability test. A P-value of < 0.05 was taken to signify statistical significance.

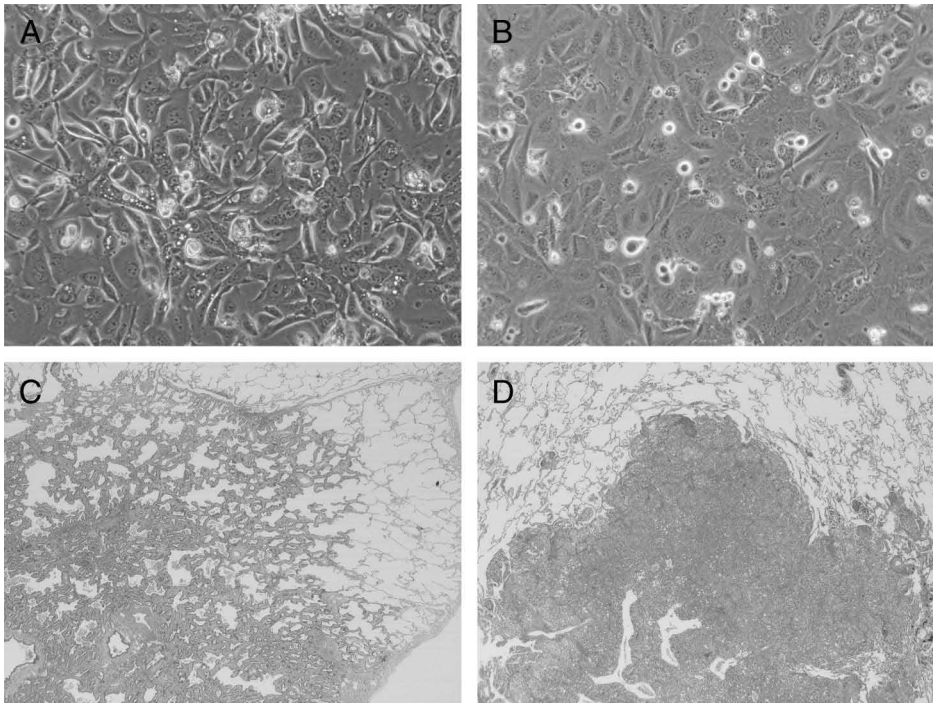

FIGURE 1. a: Phase-contrast microphotograph of PL16T which is a immortalized cell line derived from atypical adenomatous hyperplasia. (original magnification x100) b: Phase-contrast microphotograph of PL16B which is a immortalized cell line derived from the resected end of bronchus of the same patient. (original magnification x100) c: Histological feature of small-sized adenocarcinoma with bronchioloalveolar carcinoma component. (case 5 in table 2) (original magnification x4) d: Histological feature of small-sized adenocarcinoma without bronchioloalveolar carcinoma component. (case 24 in table 2) (original magnification x4)

RESULTS

We initiated our research with a pair of cell lines, PL16T established from atypical adenomatous hyperplasia and PL16B established from normal bronchial epithelium of the same patient (Fig. 1a & 1b). The MCA-SSH genome-scanning approach first involves isolation of CpG-rich *Sma* I-digested fragments and generation of a methylation-specific library by the MCA method reported previously by Toyota et al.[13] Then, using PL16T as the tester and PL16B as the driver, we performed SSH to exclude sequences common to the tester and driver populations. Finally, by T/A cloning and differential screening, 229 clones from a total of 2134 clones were selected for sequence analysis.

Among the 229 clones, 190 DNA fragments could be sequenced with the ABI PRISM 310 Genetic Analyzer and their adjacent sequences were obtained by a BLAST search. From the 190 DNA fragments, 29 genes could be identified (Table 1). In these 29 DNA fragments, 15 had a genomic region that met the criteria for a CpG island (minimum length, 200bp; GC content, >50%; CpG/GpC, >0.5). We then used quantitative RT-PCR to analyze the relative expression levels between PL16T and PL16B. Among the 15 genes, apoptotic chromatin condensation inducer 1 (ACIN1) showed significant difference of its expression level between PL16T and PL16B and we finally selected the gene for further analysis.

The genome structure of the ACIN1 gene is shown in Fig. 2. The size of the captured DNA fragment was 670 base pairs, including exon 17, intron 17, exon 18, and intron 18. The methylation status of the locus at the 5′ end of the cloned DNA fragment of ACIN1 was examined by the bisulfite sequencing technique in the 37 paired surgical specimens of

TABLE 1. List of the 29 genes identified in the DNA fragments isolated by MCA-SSH

Gene	Full name	Location of the DNA fragment	CpGI*	Chromosomal position
ABR	active BCR-related gene	intron2	yes	17p13.3
ACIN1	apoptotic chromatin condensation inducer 1	exon17 - intron17 - exon18 – intron18	yes	14q11.2
ARHGAP10	rho-GTPase activating protein 10	intron9	no	4q31.23
Bmp8A	bone morphogenetic protein 8 a	intron6	no	1p34
CMIP	C-maf-inducing protein	intron1	yes	16q23
C20orf9 frame	chromosome 20 open reading frame 9	intron6	no	6p21.3
DKFZP434H132	DKFZP434H132 protein	exon1	yes	15q23
DUX4	double homeobox 4	exon1	yes	4q35.2
IGFL2	insulin growth factor-like family member 4	intron1	no	19q13.22
KCNJ14	potassium inwardly rectifying channel, subfamily J, member 14	intron1	yes	19q13
KIAA	KIAA0676 protein	intron4	no	5q35.3
MSH5	mutS homolog 5 (E. coli)	intron7	yes	6p21.3
MVD	mevalonate (diphospho) decarboxylase	intron8	no	16q24.3
NPTX1	neuronal pentraxin 1	exon5 - 3' untranslated region	yes	17q25.1-25.2
PKD1	polycystic kidney disease 1 (autosomal dominant)	exon12 - intron12	yes	16q13.13
PLEKHG2	pleckstrin homology domain containing, family G member 2	intron13	no	6q25.1
POLM	polymerase (DNA directed), mu	intron7	no	7p13
PRKAR1B	protein kinase cAMP-dependent, regulatory type 1	intron6	no	7pter-p22
RASA3	RAS p21 protein activator 3	intron9	no	13q34
SALL4	sal-like 4 (Drosophila)	intron1	no	20q13.13-q13.2
SH3BP2	SH3-domain binding protein 2	exon3 - intron3 - exon4	yes	4p16.3
SLC16A1	solute carrier family 16 (monocarboxylic acid transporters) member 1	intron2	yes	1p12
SLC39A11	solute carrier family 39 (metal ion transporters) member 11	intron6	yes	17q24-.q25.1
SPG7	spastic paraplegia 7, paraplegin	3' untranslated region	yes	16q24.3
STX8	syntaxin 8	intron6	no	17p.12
TANC	TPR domain, ankyrin-repeat and coiled-coil-containing	intron	no	2q24.2
TRIM56	tripartite motif-containing 56	exon1	yes	7q22.1
USP18	ubiquitin specific protease18	intron3	yes	22q11.21
WNT7B	wingless-type MMTV integration site family member 7B	intron2	no	22q13

* The relaxed criteria described previously were used for finding CpG islands: minimum length 200 bp; G + C > 50%; observed CpG/expected CpG > 60%.

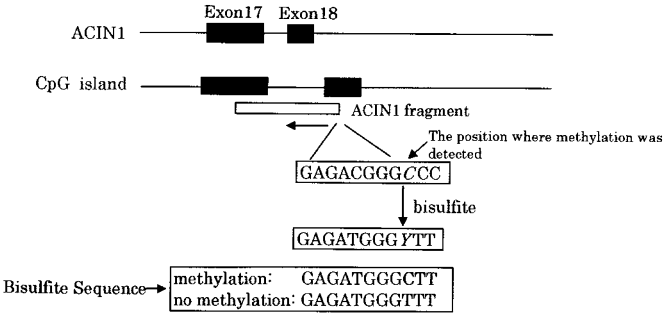

FIGURE 2. Genome structure of the ACIN1 gene and the locations of CpG islands (full black boxes). The position of the DNA fragment isolated by MCA-SSH is indicated by the open box. *C* denotes the cytosine residues at the 5′ end of the isolated DNA fragment, with unknown methylation status. During sodium bisulfite DNA modification, unmethylated cytosine residues are converted to thymine, whereas methylated cytosine residues are retained as cytosine at the CpG site. After bisulfite DNA modification, the DNA fragments were amplified by PCR using appropriate primers and their sequences were determined with an automated sequencer.

early lung adenocarcinoma and the adjacent normal tissues. These specimens included 21 cases of adenocarcinoma with BAC and 16 cases of adenocarcinoma without BAC (Fig. 1c & 1d). The pathologic stage and clinical characteristics of the cases are shown in Table 2 and 3. After bisulfite modification, three to five clones from each specimen were sequenced and examined for methylation status. We judged the specimen methylated if all clones were methylated. As shown in Table 2 and Table 3, 30 of 37 (81%) tumor specimens were methylated but only 9 of 37 (24%) adjacent noncancerous tissues were methylated ($P < 0.05$). All the cases in which the adjacent noncancerous tissue was judged as methylated also showed methylation in the tumor tissue (Table 2). There was no correlation between pathologic stage and/or clinical characteristics of the adenocarcinoma and methylation status (Table 2). Interestingly, the frequency of methylated status in the surrounding noncancerous tissue of the small-sized lung adenocarcinomas without BAC was significantly ($P < 0.05$) higher than that of adenocarcinomas with BAC (Table 3). However, there was no difference in tumor methylation status between small-sized adenocarcinomas with BAC and those without BAC (Table 3). Among the adenocarcinoma cases with BAC component, the BAC/total tumor ratio did not

TABLE 2A. Summary of ACIN1 gene methylation and Clinicopathologic Features in Patients with Small-Sized Adenocarcinoma with bronchioloalveolar carcinoma component of the lung

Classification of small-sized adenocarcinoma	Case number	Pathologic stage[a]	Gender	Age (years)	SI	Diameter (mm)	BAC/total[b] tumor area (%)	WHO classification[c]	ACIN1[d] M(T)	ACIN1[d] M(N)
	1	IA	F	56	520	13	100	pure BAC	2/4	2/4
	2	IA	F	57	0	12	100	pure BAC	5/5	4/4
	3	IA	F	80	900	20	60	mix BAC+acinar	4/5	4/5
	4	IA	F	77	0	9	100	pure BAC	4/4	1/4
	5	IA	F	49	0	20	100	pure BAC	5/5	4/5
	6	IA	F	58	0	10	100	pure BAC	5/5	3/4
	7	IIA	F	68	0	10	50	mix BAC+acinar	5/5	3/5
Adenocarcinoma	8	IA	M	68	1620	20	30	mix BAC+acinar	5/5	3/5
with BAC	9	IIIA	F	47	0	17	70	mix BAC+acinar	4/5	2/5
	10	IA	M	52	0	15	80	mix BAC+acinar	4/4	3/4
	11	IA	F	58	0	15	60	mix BAC+acinar+papi	4/4	2/4
	12	IA	F	56	4	20	50	mix BAC+acinar	4/4	3/4
	13	IA	F	47	0	15	80	mix BAC+acinar+papi	3/3	1/4
	14	IA	M	73	1100	20	40	mix BAC+acinar	4/4	3/4
	15	IB	M	56	0	15	60	mix BAC+acinar	4/5	4/5
	16	IA	M	78	550	14	80	mix BAC+acinar	5/5	2/5
	17	IIIA	M	55	700	20	30	mix BAC(mucinous)+acinar	5/5	3/5
	18	IA	M	65	1000	11	90	mix BAC +acinar+solid	5/5	3/5
	19	IB	M	72	1120	14	30	mix BAC +papi+acinar	5/5	3/5
	20	IA	M	81	0	16	30	mix BAC +acinar+solid	5/5	5/5
	21	IB	M	57	900	20	30	mix BAC +acinar+solid	5/5	2/4

[a]Pathologic stage: I (IA and IB), II (IIA and IIB), III (IIIA and IIIB); SI: smoking index: (the number of cigarettes smoked/day) x years.
[b]BAC: bronchioloalveolar carcinoma, components of BAC in the cases were non-mucus except case number 18.
[c]mix: mixed adenocarcinoma; acinar: acinar adenocarcinoma; papi: papillary adenocarcinoma.
[d]M: methylate cytosine, five clones of the isolated DNA fragment were sequenced by bisulfite sequencing in 37 cases of lung adenocarcinoma (T) and the surrounding normal tissue(N). The frequency of methylation in 5′ end CpG sites of isolated DNA fragments of the ACIN1 gene were showed as the number of clones methylated /the number of clones examined.

TABLE 2B. Summary of ACIN1 gene methylation and Clinicopathologic Features in Patients with Small-Sized Adenocarcinoma without bronchioloalveolar carcinoma component of the lung

Classification of small-sized adenocarcinoma	Case number	Pathologic stage	Gender	Age (years)	SI	Diameter (mm)	BAC/total tumor area	WHO classification	ACIN1 M(T)	ACIN1 M(N)
•	22	IA	F	76	0	20	0	mix papi+acinar	4/4	4/4
	23	IIA	F	77	0	17	0	mix acinar+solid	5/5	3/4
Adenocarcinoma	24	IA	M	62	0	13	0	mix acinar+solid	5/5	3/5
without BAC	25	IA	M	66	440	18	0	mix acinar+solid	5/5	5/5
	26	IA	M	61	1480	15	0	mix solid+acinar+papi	5/5	5/5
	27	IIIB	M	46	0	20	0	mix solid+acinar	5/5	4/5
	28	IA	M	60	1600	18	0	mix solid+acinar+papi	5/5	5/5
	29	IIIA	M	71	2700	18	0	mix solid+acinar	4/5	4/5
	30	IA	M	63	860	15	0	papi	5/5	3/5
	31	IA	M	82	840	18	0	solid	4/4	4/5
	32	IIIA	M	46	0	13	0	papi	5/5	3/5
	33	IA	F	74	0	16	0	acinar	4/5	2/5
	34	IA	F	59	0	17	0	papi	4/5	3/5
	35	IA	M	62	1080	18	0	solid	5/5	5/5
	36	IA	M	59	430	15	0	acinar	5/5	5/5
•	37	IA	M	69	980	15	0	solid	5/5	5/5

[a]Pathologic stage: I (IA and IB), II (IIA and IIB), III (IIIA and IIIB); SI: smoking index: (the number of cigarettes smoked/day) x years.
[b]BAC: bronchioloalveolar carcinoma, components of BAC in the cases were non-mucus except case number 18.
[c]mix: mixed adenocarcinoma; acinar: acinar adenocarcinoma; papi: papillary adenocarcinoma.
[d]M: methylate cytosine, five clones of the isolated DNA fragment were sequenced by bisulfite sequencing in 37 cases of lung adenocarcinoma (T) and the surrounding normal tissue(N). The frequency of methylation in 5′ end CpG sites of isolated DNA fragments of the ACIN1 gene were showed as the number of clones methylated /the number of clones examined.

influenced the tumor methylation status or the noncancerous tissue methylation status (Table 2).

We performed quantitative RT-PCR to measure expression levels of the ACIN1 gene in lung adenocarcinoma and the adjacent normal tissue, using three specimens of lung adenocarcinoma with BAC component and three specimens of lung adenocarcinoma without BAC component (Fig. 3). All the tumor tissues examined were methylated cases about ACIN1 (Table 1), but there were no significant correlations between the ACIN1 gene expression levels of lung adenocarciomas and their methylation status.

DISCUSSION

PL16T and PL16B are immortalized cell lines derived from a precancerous lesion of lung adenocarcinoma and from normal bronchial epithelium of the same patient.[19] Therefore, they represent a useful tool for investigation of early epigenetic changes in lung adenocarcinoma. In this study, we examined methylation status in these cell lines by a combination of MCA and SSH methods. We established that the ACIN1 gene shows significant differences in methylation status and expression level between PL16T and PL16B. The ACIN1 (apoptotic chromatin condensation inducer 1) gene encodes the protein Acinus, which is essential for apoptotic chromatin condensation *in vitro* and is also important in the induction of apoptotic chromatin condensation in cells.[24] No information about the hyper- or hypo-methylation of ACIN1 in cancer was previously available. We examined methylation status at the same locus in paired tissue specimens from 37 cases of small lung adenocarcinoma and the adjacent

TABLE 3. Frequency of methylation in lung adenocarcinoma and its surrounding noncancerous tissue

	Cancerous legion			Non-cancerous region[b]		
Methylation	T with BAC[a]	T without BAC[a]	Total	T with BAC	T without BAC	Total
+	17 (81%)	13 (81%)	30 (81%)	2 (10%)	7 (44%)	9 (24%)
−	4 (19%)	3 (19%)	7 (97%)	19 (90%)	9 (56%)	28 (76%)
Total	21	16	37	21	16	37

+: Methylated cytosine residues were found in the 5′ end CpG sites of the isolated DNA fragment in all of the clones examined; −: Unmethylated cytosine residues were found in at least one of the clones examined.
[a]T with BAC: tumor with BAC component; T without BAC: tumor without BAC component.
[b]The difference between T with BAC and T without BAC in the frequency of methylation in the surrounding noncancerous region of lung adenocarcinoma was statistically significant:, $P < 0.05$

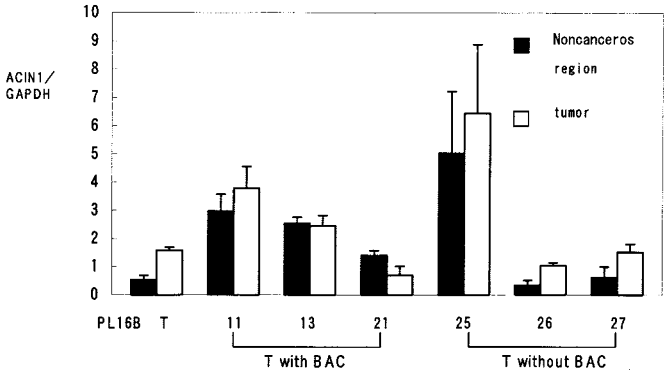

FIGURE 3. Expression of the ACIN1 gene. Expression levels were analyzed by quantitative RT-PCR in the pair of cell lines (PL16T and PL16B), in three surgical specimens of lung adenocarcinoma with BAC component (case numbers 11, 13, 21; see Table 2) and in three specimens of adenocarcinoma without BAC component (case numbers 25, 26, 27; see Table 2) together with their normal adjacent tissues. T with BAC: adenocarcinoma with bronchioloalveolar carcinoma component; T without BAC: adenocarcinoma without bronchioloalveolar carcinoma component.

normal tissue using bisulfite sequencing technique. The frequency of methylation of ACIN1 in tumors was higher than in the surrounding noncancerous tissue, both in tumors with BAC component (21 patients, average age 61 years) and in tumors without BAC component (16 patients, average age 64.6 years) (Tables 2 and 3). But there were no significant differences of the frequencies of hypermethylation of ACIN1 between tumors with BAC and tumors without BAC. No effect of gender, age or smoking index on methylation status was also found. Our observation suggests that hypermethylation of this locus is a common event in the early stages of lung adenocarcinoma, regardless of the histological subtype of lung adenocarcinoma, such as adenocarcinoma derived from AAH or BAC and *de novo* adenocarcinoma without BAC component.

Clones from the surrounding normal tissue showed various extents of methylation (Table 3), and we observed that the methylation frequency of ACIN1 in normal tissue was higher in cases of adenocarcinoma without BAC component than in cases of adenocarcinoma with BAC component (Tables 2 and 3). Two possible explanations may account for this observation. First, there may be diversity of cellular methylation status in normal lung tissue, and the methylation status of ACIN1 in normal tissue may determine the histological type of the adenocarcinoma. Secondly, the background methylation status of the normal lung tissue may reflect degree of exposure to carcinogens such as cigarette smoke or diesel exhaust.

The expression analysis of ACIN1 gene in this study showed no correlation between the expression level of ACIN1 and its methylation status (Fig. 3). Usually, the methylation of CpG islands in promoter region was reported to influence the expression level of the gene such as $p16^{INK4A}$.[25] However, the methylated locus of ACIN1 gene was located on the CpG islands in the exon-intron region. Current evidence suggests that methylation of coding sequences or introns does not directly silence the expression level of ACIN1 gene. Therefore, the differences of ACIN1 gene expression between PL16T and PL16B could not be interpreted by the methylation at the locus detected in this study. The potential methylation site of ACIN1 that may influence expression is speculated to be located on the CpG island in the promoter region of the gene. We believe that the results of this study indicated that we detected one of the hyper-methylated sites in the course of pulmonary adenocarcinogenesis and we may use the results to screen the high-risk population for non-BAC type adenocarcinoma.

In this study, we found hypermethylation of the ACIN1 gene in not only early stage lung adenocarcinoma, but also some non-tumorous regions of adenocarcinoma cases. These results suggested that the methylation of ACIN1 gene is one of the early events in the lung adenocarcinoma development and also suggested the possibility to identify persons with high risk of some type of lung adenocarcinoma. Nevertheless, the role of methylation status in the development and malignant progression of lung adenocarcinoma requires clarification.

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